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(54) **PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS.**

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Chemical abstracts, volume 102, n 7, 18 February 1985, (Columbus, Ohio, US), H.M. Kronenberg et al "Studies of parathyroid hormone secretion using recombinant DNA technology", see page 109, abstract 55590j, Int, Congr. Ser, Excerpta Med. 1984, 619 (Endocr. Control Bone Calcium Metabl. vol. 8A), 217-20 (Eng)

Experientia, volume 39, n 6, June 1983, Birkhäuser Verlag (Basel, CH), W. Born et al "Expression and processing of human preproparathyroid hormone in Escherichia coli", see 659

Chemical abstracts, volume 96, n 13, 29 March 1982 (Columbus, Ohio, US) G.N. Hendy et al "Nucleotide sequence of cloned cDNAs encoding human preproparathyroid hormone", see abstract 98734x & Proc. Natl. Acad. Sci. USA, 1981, 78(12), 7365-9 (Eng.)

Proc. Natl. Acad. Sci. USA, volume 80, April 1983, T.J. Vasicek et al "Nucleotide sequence of the human parathyroid hormone gene", pages 2127-2131

Chemical abstracts, volume 105, n 1, 7 July 1986 (Columbus, Ohio, US) W.L. Sung et al "Hybrid gene synthesis : its application to the assembly of DNA sequences encoding the human parathyroid hormones and analogs", see page 151, abstract 1533z & Biochem. Cell Biol. 1986, 64(2), 133-8 (Eng.)

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FIELD OF THE INVENTION

This invention relates to genetically engineered microorganisms containing DNA coding for human preproparathyroid hormone.

BACKGROUND OF THE INVENTION

A number of proteins and peptides that are normally synthesized by mammalian cells have proven to have medical, agricultural and industrial utility. These proteins and peptides may be of different molecular size and have a number of different functions, for example, they may be enzymes, structural proteins, growth factors and hormones. In essence both proteins and peptides are composed of linear sequences of amino acids which form secondary and tertiary structures that are necessary to convey the biological activity. Human parathyroid hormone has a relatively small molecular weight, which has made it possible to synthesize the peptide chemically by the sequential addition of amino acids. Thus, parathyroid hormone is commercially available, but in very small quantities at high cost. As a result, there is no human parathyroid hormone available at a reasonable price to supply the many potential medical, agricultural and industrial applications.

During the past ten years, microbiological techniques employing recombinant DNA have made it possible to use microorganisms for the production of species-different peptides. The microorganism is capable of rapid and abundant growth and can be made to synthesize the foreign product in the same manner as bacterial peptides. The utility and potential of this molecular biological approach has already been proven by microbiological production of a number of human proteins that are now available for medical and other uses.

Parathyroid hormone (PTH) is one of the most important regulators of calcium metabolism in mammals and is also related to several diseases in humans and animals, e.g. milk fever, acute hypocalsemia and otherwise pathologically altered blood calcium levels. This hormone therefore will be important as a part of diagnostic kits and will also have potential as a therapeutic in human and veterinary medicine.

The first synthesis of DNA for human preproparathyroid hormone was described by Hendy, G.N., Kronenberg, H.M., Potts, Jr. J.T. and Rich, A., 78 Proc. Natl. Acad. Sci. 7365-7369 (1981). DNA complementary in sequence to PTH mRNA was synthesized and made double stranded (Hendy et al., supra). This cDNA was cloned in pBR 322 DNA and E. coli 1776 was transfected. Of the colonies with correct antibiotic resistance, 23 out of 200 clones were identified as containing specific human PTH cDNA inserts. However, none of the 23 human PTH clones contained the full length insert (Hendy et al., supra). Later, Born et al. (Experientia 39, 659, 1983) constructed vectors for expression of the cDNA of human prepro-PTH in bacteria. Whereas correctly processed HPTH could be detected upon disruption of the cells, soluble HPTH could not be found in the periplasmic space. Breyel, E., Morelle, G., Auf'mkolk, B., Frank, R., Blocker, H. and Mayer, H., Third European Congress on Biotechnology, 10-14 September 1984, Vol. 3, 363-369, described the presence of the human PTH gene in a fetal liver genomic DNA library constructed in the phage Charon 4A. A restriction enzyme fragment of the PTH gene was recloned and transfected into E. coli.

However, the work of Breyel et al., supra, demonstrated that E. coli degrades human PTH. Thus, a microorganism which shows a stable production of intact human parathyroid hormone has so far not been described. Further, secretion of parathyroid hormone has never before been obtained with yeasts, even though WO 84/01173 describes a method for the production of mature proteins such as HPTH in yeasts. However, the few HPTH molecules, which were secreted, did not exhibit biological activity to a significant extent.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a plasmid for insertion in yeast containing DNA coding for parathyroid hormone. It is also an object of the present invention to provide a transformed yeast containing DNA coding for parathyroid hormone, including human parathyroid hormone (HPTH), and from which transformed yeast, parathyroid hormone may be obtained.

Other objects and advantages of the present invention will became apparent as the description thereof proc eds.

In satisfaction f th foregoing objects and advantages, there is provided by the present inv ntion a n v l plasmid for insertion in yeast, containing DNA coding for human preproparathyroid h rm n . The

plasmid for insertion into yeast f the present invention is distinguishable ov r prior art plasmids, for xampl as described in Hendy et al., supra, in that th plasmid of th present invention contains a double start codon at the 5' end of the DNA coding for preproparathyroid hormone. The presence of the doubl start codon may cause a production microorganism transformed with a plasmid containing this cDNA to produce preproparathyroid hormone at an increased rate and in an improved yield over prior art transformed microorganisms.

There is provided by the present invention a plasmid for insertion in yeast containing DNA coding for parathyroid hormone. In a preferred embodiment, this plasmid is prepared by recloning the plasmid for insertion in E. coli described above. Finally, the invention provides a yeast transformed by said plasmid for insertion in yeast such that the yeast produces and secretes parathyroid hormone. Thus, the invention provides a method by which parathyroid hormone may be isolated from yeast culture medium. In a preferred embodiment, the transformed yeast is Saccharomyces cerevisiae. In another preferred embodiment, the parathyroid hormone is human parathyroid hormone.

Samples of pSSHPTH-10, E. coli transformed therewith, pSSαLX5-HPTH1 and Saccharomyces cerevisiae transformed therewith were deposited in the American Type Culture Collection in Rockville, Maryland on September 29, 1988, under the provisions of the Budapest Treaty. The samples have been accorded the following deposit numbers:

Transformed E. coli containing pSSHPTH-10: ATCC 67223.
pSSHPTH-10: ATCC 40267.
Transformed S. cerevisiae containing
pSSαLX5-HPTH1: ATCC 20821.
pSSαLX5-HPTH1: ATCC 40266.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows all possible variations of the DNA sequence coding for human preproparathyroid hormone.

Figure 2 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10.

Figure 3 shows a DNA Sequence coding for human preproparathyroid hormone and having a double start codon at the 5' terminal end with flanking sequences in which are shown all possible variations of the DNA which may be present on the plasmid of the present invention.

Figure 4 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10 with flanking sequences.

Figure 5 shows the actual amino acids sequence of the human preproparathyroid hormone for which the DNA sequence in clone pSSHPTH-10 codes.

Figure 6 shows the composition of the recombinant plasmid pSSHPTH-10.

Figure 7 shows a map of pALX4.

Figure 8 shows the construction of pαLX5 from pL4 and pMFα1-1.

Figure 9 shows the construction and schematic drawing of pSSαLX5-HPTH1.

Figure 10 shows the sequence of the MFα1-HPTH fusion gene with all possible combinations of the DNA coding for HPTH.

Figure 11 shows the sequence of the MFα1-HPTH fusion gene.

Figure 12 shows an electrophoresis plate showing the human parathyroid hormone produced and secreted by yeast and recovered from the yeast culture medium.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As indicated above, the present invention is directed to a plasmid for insertion into yeast which contains DNA coding for parathyroid hormone and which is derived from the plasmid pSSHPTH-10 shown in Fig.6. Finally, the invention is directed to a transformed yeast from which parathyroid hormone may be recovered.

The invention further provides methods of producing and isolating the plasmids and transformed microorganisms. Poly(A) selected RNA was isolated from human parathyroid adenomas collected immediately after surgery. The poly(A) RNA was enriched for correct size mRNA by ultracentrifugation through sucrose gradients. Preproparathyroid hormone of correct molecular weight was translated in vitro from this size fractionated poly(A) RNA as judged by sodium dodecylsulphate polyacrylamide gel electrophoresis after immun precipitation with antiparathyroid antiserum. The specific messenger RNA for the human PTH was used as template for complementary DNA synthesis using oligo d(T)18 as a primer and avian

myoblastosis virus reverse transcriptase. After removal of the RNA templates by alkali hydrolysis, the second strand complementary DNA was synthesized by incubating th purified first strand DNA in th presence of th Klenow fragment of E. coli DNA polymerase I. The double stranded complementary DNA was made blunt ended by the action of Aspergillus oryzae single strand specific endonuclease S1 and complementary DNA longer than 500 base pairs was isolated after neutral sucrose gradient centrifugation. Approximately 20 bases long d(C)-tail protrusions were enzymatically added to the 3' ends of the cDNA. This modified complementary DNA was annealed to restriction endonuclease PstI cleaved and G-tailed vector pBR 322. Resulting recombinant plasmid DNA's were transformed into E. coli KI2 BJ 5183. Positive transformants were analysed for by colony hybridization using two different synthetic deoxyribo-oligonucleotides which covered the N-terminal coding region as well as the 3' noncoding part of the hormone mRNA sequence, respectively. Six out of 66 clones that were positive for both probes were submitted for detailed analysis by restriction endonuclease mapping showing that they all were identical except for some size heterogenity at the regions flanking the start codon and the XbaI site 3' for the stop codon. One clone, pSSHPTH-10, was subjected to DNA sequence analysis revealing a 432 nucleotide long human parathyroid hormone complementary DNA sequence inserted in the PstI site of pBR 322. The entire cDNA sequence was found to be identical to the sequence previously described by Hendy, et al., supra, except for a five base pair deletion in front of the start codon.

Figure 2 shows the human preproparathyroid hormone DNA sequence of pSSHPTH-10. This may be compared with Figure 1, which shows all possible variations of the DNA sequence for human preproparathyroid hormone without the 5' double start codon. Figure 3 shows a DNA sequence coding for human preproparathyroid hormone and having a double start codon at the 5' terminal end with flanking sequences in which are shown all possible variations of the DNA which may be present on the plasmid of the present invention.

A plasmid coding for human preproparathyroid hormone is pSSHPTH-10. the DNA sequence of which, including the flanking sequence, is shown in Figure 4.

Figure 5 shows the DNA sequence coding for preproparathyroid hormone in pSSHPTH-10 with flanking sequences showing the corresponding amino acid sequence of preproparathyroid hormone.

The invention provides a plasmid for insertion into yeast containing DNA coding for parathyroid hormone.

Fig. 10 shows a partial DNA sequence for the plasmid for insertion into yeast in which: Nucleotide nos.1-173 makeup the MFα 1 promoter region and 5' noncoding sequence. 174-440 is the MFα1 N-terminal coding sequence. 441-695 is an HPTH sequence. 696-726 is an HPTH 3' noncoding sequence from pSSHPTH-10. 727-732 is from pUC19. 733-874 is MFα1 3' noncoding sequence and transcriptional termination signal.

The parathyroid hormone may be human or animal parathyroid hormone, for example pig or bovine parathyroid hormone. The plasmid for insertion in yeast of the present invention may be recloned from plasmids containing DNA coding for human or animal parathyroid hormone.

Figure 11 shows theNucleotide sequence of the MFα1-HPTH fusion gene from pSSαLX5-HPTH1. Nucleotide nos. 1-173 makeup the MFα1 promoter region and 5' noncoding sequence. 174-440 is the MFα1 N-terminal coding sequence. 441-695 is the HPTH sequence obtained from pSSHPTH-10. 696-726 is an HPTH 3' noncoding sequence from pSSHPTH-10. 727-732 is from pUC19. 733-874 is MFα1 3' noncoding sequence and transcriptional termination signal.

In a preferred embodiment, the plasmid for insertion in yeast contains DNA coding for human parathyroid hormone. As shown in the following examples, the HPTH sequence from pSSHPTH-10 has been recloned and inserted in specially designed vectors for expression in Saccharomyces cerevisiae.

pSSHPTH-10 was digested to form a $_{288}$ bp BglII-XbaI fragment. This fragment was then subcloned into pUC19 between the BamHI and XbaI sites. The subclone was then digested with Dpn I, and the largest resulting fragment was isolated. The said fragment was then digested with SalI.

The plasmid pSSαLX5-HPTH1 that in yeast MATα cells leads to the expression and secretion of PTH was constructed in three stages:

1. Construction of the yeast shuttle vector pL4 (which replicates in both E. coli and Saccharomyces cerevisiae).
2. Cloning of a DNA fragment containing the yeast mating pheromone MFα1 gene and its insertion into the yeast shuttle vector to make the pαLX5 vector.
3. Insertion of a DNA fragment fr m th coding region of the HPTH gene of pSSHPTH-10 into pαLX5 in reading fram with the prepr part of the MFα1 gene, thereby producing the vector pSSαLX5-HPTH1.

The shuttle vector pL4 was constructed by inserting into pJDB207, an EcoRI-AvaII fragm nt containing the ADHI promoter isolated from pADH040. A SphI fragment was then deleted, r sulting in a plasmid

pALX1. Th PstI Site in the β-lactamase gene was deleted and the plasmid was partially digested with PvuI and BglI and ligated to a PvuI BglI fragment of pUC8, to form pALX2. After a further oligonucleotide inserti n, the plasmid was digested with HindIII and religated t form pALX4.

Total yeast DNA from the Y288C strain was digested with EcoRI, and the 1.6-1.8 kb fragments isolated. These were ligated to EcoRI-cleaved pBR322, and E. coli was transformed. The clones were screened for MFα1 inserts by oligonucleotide hybridization. The DNA selected thereby was then used to transform E. coli. The resulting plasmid pMFα1-1 was digested with EcoRI, made blunt ended by Klenow enzyme, and then digested with BglII. The MFα1 fragment was isolated, and ligated to pL5 (digested with BamHI, made blunt ended with Klenow enzyme, and digested with BglII) to yield pαLX5.

In order to insert the human PTH cDNA fragment into pαLX5, the pαLX5 was digested with HindIII, creating sticky ends and the site was made blunt ended with the DNA polymerase I Klenow fragment and dNTP. The pαLX5 was then digested with SalI to create a sticky ended DNA complementary to the SalI digested human PTH fragment described above.

The SalI digested human PTH fragment was then inserted into the SalI digested pαLX5. The resulting plasmid pSSαLX5-PTH is shown in Figure 9.

pSSαLX5-PTH was then inserted into yeast, thereby transforming yeast so that the yeast produces and secretes human parathyroid hormone. In a preferred embodiment, the transformed yeast is Sacchromyces cerevisiae. An electrophoresis plate showing the human parathyroid hormone from the yeast culture medium is shown at Figure 12.

Although the method for making the plasmid for insertion in yeast by recloning pSSHPTH-10 is shown in detail, this method is shown to illustrate the invention, and the invention is not limited thereto. The method may be applied to a variety of other plasmids containing DNA coding for human or animal PTH to produce the plasmid for insertion in yeast of the present invention.

The plasmids of the present invention and transformed microorganisms were produced as set forth in the following examples.

EXAMPLE I

Isolation of mRNA and synthesis of complementary DNA (cDNA) of human parathyroid hormone.

Starting material for the invention was parathyroid adenomas obtained from patients by surgery. The parathyroid tissue was placed on dry ice directly after removal and transported to a laboratory for preparation of RNA. The frozen tissue was homogenized with an ultra Turax homogenizer in the presence of 4 M Guanidinium thiocyanate and the RNA content was recovered by serial ethanol precipitations as described by Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J., 18 Biochemistry 5294-5299 (1979). The RNA preparation was applied to oligo d(T) cellulose affinity chromatography column in order to enrich for poly(A) containing mRNA. The poly(A) rich RNA was further enriched for parathyroid hormone (PTH) mRNA sized RNA by ultracentrifugation through a 15-30% linear sucrose gradient. The resulting gradient was divided into 25 fractions and every third fraction was assayed for PTH mRNA content by in vitro translation followed by immunoprecipitation with anti PTH antiserum (Gautvik, K.M., Gautvik, V.T. and Halvorsen, J.F., Scand. J. Clin. Lab. Invest. 43, 553-564 (1983)) and SDS-polyacrylamide gel electrophoresis (Laemmeli, U.K., 227 Nature 680 (1970)) . The RNA from the fractions containing translatable PTH mRNA was recovered by ethanol precipitation.

This RNA, enriched for PTH mRNA, was used as a template for cDNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase for catalysis of the reaction (Maniatis, T., Fritsch, E.F. and Sambrook, J., Molecular Cloning pp. 230-243 (1982)). After first strand synthesis, the RNA templates were removed by alkali hydrolysis. The second strand cDNA was synthesized by incubating the purified first strand cDNA in the presence of the Klenow fragment of E. coli DNA polymerase I (Maniatis, supra). This in vitro synthesized double stranded cDNA was made blunt ended by the action of Aspergillus oryzae single strand specific endonuclease S1 (Maniatis, supra). The blunt ended double stranded cDNA was size fractionated over a 15-30% neutral sucrose gradient. The size distribution of each fraction was estimated by agarose gel electrophoresis together with known DNA fragment markers. Fractions containing cDNA larger than approximately 500 base pairs were pooled and the cDNA content was collected by ethanol precipitation.

EXAMPLE 2

Cloning of cDNA PTH in plasmid pBR 322 and transformation of E. coli K12 BJ5183.

An approximate 20 base long d(C)-tail protrusion was enzymatically added to the 3' ends of the cDNA by the action of terminal deoxynucleotidyl transferase (Maniatis, supra). The d(C)-tailed cDNA was annealed to restriction endonuclease PstI cleaved and d(G)-tailed vector pBR322 and the resulting recombinant plasmid DNA's were transformed into E. coli K12 BJ 5183 cells which were made competent by the method of Hanahan, D., 166 J. Mol. Biol. 557-580 (1983). A total of 33,000 transformants were analyzed for PTH cDNA content by colony hybridization (Hanahan, D. and Meselson, 10 Gene 63 (1980)).

Two to three thousand transformants were plated directly on each 82 mm diameter nitrocellulose filter, placed on top of rich medium agar plates containing tetracycline, and incubated at 37 degrees Centigrade until approximately 0.1 mm diameter colonies appeared. Duplicate replicas of each filter was obtained by serial pressing of two new filters against the original filter. The replica filters were placed on top of new tetracycline containing agar plates and incubated at 37 degrees Centigrade until approximately 0.5 mm diameter colonies appeared. The master filter with bacterial colonies was kept at 4 degrees Centigrade placed on top of the agar plate and the duplicate replica filters were removed from the agar plates and submitted to the following colony hybridization procedure.

EXAMPLE 3

Characterization of bacterial clones containing recombinant PTH cDNA and of the DNA sequence of clone pSSHPTH-10.

The cells in the respective colonies were disrupted in situ with alkali and sodium chloride leaving the DNA content of each bacterial clone exposed: The procedure allows the DNA to bind to the filter after which it was neutralized with Tris-buffer and dried at 80 degrees Centigrade. The majority of cell debris was removed by a 65 degree Centigrade wash with the detergent sodium dodecylsulphate (SDS) and sodium chloride leaving the DNA bound to the filters at the position of the former bacterial colonies. The filters were presoaked in 6xSSC (0.9M NaCl, 0.09M Na-citrate), 1x Denhart's solution (0.1 g/ml Ficoll, 0.1 g/ml polyvinyl pyrrolidone, 0.1 g/ml bovine serum albumin),

100µg/ml herring sperm DNA, 0.5% SDS and 0.05% sodium pyrophosphate for two hours at 37 degrees Centigrade (Woods, D.E., 6 Focus No. 3. (1984)).

The hybridization was carried out at 42 degrees Centigrade for 18 hours in a hybridization solution (6x SSC, 1x Denhart's solution, 20µg/ml tRNA and 0.05% sodium pyrophosphate) supplemented with 32P-labelled DNA probe (Woods, supra).

The DNA used as a hybridization probe was one of two different synthetic deoxyribo oligonucleotides of which the sequences were deduced from the published human PTH cDNA sequence of Hendy, et al., supra. The first probe was a 24-mer oligonucleotide originating from the start codon region of the human preproPTH coding sequence having a nucleotide sequence reading TACTATGGACGTTTTCTGTACCGA. The second oligonucleotide was a 24-mer spanning over a cleavage site for the restriction endonuclease XbaI located 31 nucleotides downstream of the termination codon and consisted of the nucleotide sequence CTCAAGACGAGATCTGTCACATCC.

Labelling was carried out by transfer of 32 P from 32 P-γ-ATP to the 5' end of the oligonucleotides by the action of polynucleotide kinase (Maxam, A.M. and Gilbert, W., 65 Methods Enzymol., 499 (1980)).

The hybridized filters were washed in 6xSSC, 0.05% sodium pyrophosphate at 42 degrees Centigrade prior to autoradiography. Sixty-six clones were found to be positive for both probes as judged from hybridization to both copies of the duplicate replica filters. All those were picked from the original filters with the stored cDNA library and amplified for indefinitive storage at -70 degrees Centigrade. Six of these were chosen for plasmid preparation and a more detailed analysis by restriction endonuclease mapping, showing that all were identical except for some size heterogenity at the regions flanking the start codon and XbaI site, respectively.

EXAMPLE 4

Clon pSSHPTH-10.

One clone, pSSHPTH-10, was subjected to DNA sequence analysis according to the method of Maxam and Gilbert, supra. The complete structure of pSSHPTH-10 is shown in Figure 6. This clone consists of a 432 base pair long PTH cDNA sequence inserted in the PstI site of pBR322 having 27 G/C base pairs at the 5' end and 17 G/C base pairs at the 3' end. The complete DNA sequence of the cDNA insert of pSSHPTH-10 is shown in Figure 4. It is identical to the sequence of Hendy, et al., supra, except for a five base pair deletion right in front of the start codon, changing the published (Hendy, et al., supra) start-stop (ATGTGAAG) signal (deletion is underlined) preceding the used start codon (ATG) to a double start signal (ATGATG).

EXAMPLE 5

Construction of the yeast shuttle vector pL4.

Before the HPTH-yeast-expression project was initiated, a family of general yeast expression vectors were developed. One of these, pL4, later was used to make pSSαLX5-HPTH1, as described below:

The plasmid pJDB207, constructed by Beggs, J.D., "Multiple-copy yeast plasmid vectors," Von Wettstein, D., Friis, J., Kielland-Brandt, M. and Stenderup, A. (Eds) Molecular Genetics in Yeast (1981), Alfred Benzon Symposium Vol. 16, 383-390, was chosen as the basis for the general expression vectors. It contains an EcoRI fragment of the yeast 2 micron DNA inserted into the pBR322 derivative pAT153. It also contains the yeast LEU2 gene. The copy number of pJDB207 in yeast cir^+ cells is very high relative to that of other plasmids and it is unusually stable after non-selective growth in a cir^+ strain. Parent, S.A., Fenimore, C.M., and Bostian, K.A. "Vector Systems for the Expression, Analysis and Cloning of DNA Sequences in S. cerevisiae," 1 Yeast 83-138 (1985); Erhart, E. and Hollenberg, C.P., "The Presence of a Defective LEU2 Gene on 2 Micron DNA Recombinant Plasmids of Saccharomyces cerevisiae is Responsible for Curing and High Copy Number," 156 J. Bacteriol 625-635 (1983). These properties are related to a partial defective promoter in the selective marker gene LEU2 (often named LEU2d, d for defective), Erhart et al., supra, which is not changed in the following constructs.

A 1260 base pair EcoRI-AvaII fragment containing the ADHI promoter was isolated from the plasmid pADH040. After a fill in reaction with the Klenow fragment of DNA polymerase I and all four dNTPs, BamHI linkers were attached and the fragment was cloned into the unique BamHI site of pJDB207. From the plasmid with the promoter in a counterclockwise direction, a 1050 base pair SphI fragment was then deleted (from the SphI site in pJDB207 to the SphI site in the promoter fragment) leaving only a single BamHI site. This plasmid was designated pALX1.

The PstI site in the β-lactamase gene of pALX1 then was eliminated without inactivating the gene. pALX1 was digested to completion with PstI and nuclease S1 to destroy the PstI site, and then subjected to a partial digestion with PvuI and BglI. At the same time, a 250 base pair PvuI BglI fragment was isolated from pUC8, Vieira, J. and Messing, J., 19 Gene 259 (1982), that contains the corresponding part of a β-lactamase gene without a PstI site. This was ligated to the partially digested pALX1. In all the ampicillin resistant clones isolated the β-lactamase gene had been restored by incorporating the pUC8 fragment. This plasmid was called pALX2.

The following oligonucleotide was purchased from Prof. K. Kleppe, University of Bergen, and inserted into the BamHI site of pALX2:

```
     BglII    *  ↓↓↓* *   HindIII
GATCAGATCTGCAGGATGGATCCAAAGCTT      ↓↓↓: initiation condon
    TCTAGACGTCCTACCTAGGTTTCGAACTAG   * : optimal ATG context
       PstI      BamHI
```

Plasmids with the proper orientation were isolated and designated pALX3.

Finally the pALX3 was digested with HindIII and religated to delete a HindIII fragment of 480 base pairs. The resulting vector is called pALX4. A map of pALX4 is shown in Figure 7.

pL4 is a derivative of pALX4 in which th ADHI pr moter is deleted. pL4 was used as basis for the insertion of ther prom ters. pALX4 was first digested with BglII and SalI. The resulting sticky ends w re

filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, followed by religati n. By this treatment, th ADHI prom ter is eliminated and the BglII site regenerated to give the vector pL4.

EXAMPLE 6

Construction of pαLX5.

The gene for the yeast mating pheromone MFα1 was first cloned by Kurjan, J. and Herskowitz, I., "Structure of a Yeast Pheromone Gene (MFα): A Putative α-Factor Precursor Contains Four Tandem Copies of Mature α-Factor", 30 Cell, 933-943 (1982). The published sequence was used to reclone the MFα1 gene. Total yeast DNA from the strain Y288C was digested with EcoRI and digestion products in the size range from 1.6 to 1.8 kb were isolated from a preparative agarose gel. These were then ligated to dephosphorylated EcoRI cleaved pBR322 and used to transform E. coli BJ5183. The resulting clones were screened for MFα1 gene inserts by hybridization to a labeled oligonucleotide of the following composition:

TGGCATTGGCTGCAACTAAAGC

DNA from purified positive clones was then used to transform E. coli JA221 from which plasmid DNA was prepared. The plasmid used in the following constructs, pMFα1-1, is shown in Figure 8.

pMFα1-1 was digested with EcoRI, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BglII. The 1.7 kb MFα1 gene fragment was isolated from an agarose gel. Before inserting it into the yeast shuttle vector, the HindIII site of pL4 was eliminated by HindIII digestion, Klenow fill-in reaction and religation to give the pL5 shuttle vector. pL5 was digested with BamHI, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BglII. After purification on gel it was ligated to the MFα1 fragment to give the expression vector pαLX5 as shown in Figure 8.

EXAMPLE 7

Construction of pSSαLX5-HPTH1

A 288 base pair BglII XbaI fragment from the pSSHPTH-10 plasmid was isolated and subcloned in pUC19 using the BamHI and XbaI site of this vector. This subclone designated pUC-HPTH, was digested with DpnI and the largest fragment isolated. This fragment was then digested with SalI and the smallest of the two resulting fragments was again isolated, yielding a sticky end on the SalI cut side and a blunt end at the DpnI cut side.

pαLX5 was digested with HindIII, filled in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with SalI. After purication from gel, it was ligated to the HPTH fragment described above. The resulting clones had the HindIII site regenerated verifying that the reading frame was correct. This plasmid called pSSα LX5-HPTH1 is shown in Figure 9 The sequence of the MFα 1-HPTH fusion gene is shown in Figure 10.

EXAMPLE 8

Expression And Secretion Of HPTH In Yeast

The yeast strain FL200 (α, ura3, leu2) was transformed with the plasmids pαLX5 and pSSαLX5-HPTH1 using the spheroplast method. One transformant of each kind was grown up in leu$^-$ medium and aliquots of the cell-free medium were analysed by SDS-PAGE developed by silver-staining (Fig. 12). Two major bands were seen in the medium from the pSSαLX5-HPTH1 transformant that were not present in the medium from the pαLX5 transformant: one band of approximately 9000 daltons, the expected size of HPTH, and one band of approximately 16000 daltons that could correspond to an unprocessed MFα1-HPTH fusion product. Both polypeptides reacted with antibodies against human PTH in a manner identical to the native hormone.

The examples are included by way of illustration, but the invention is not limited thereto. While the above examples are directed to providing a S. cerevisiae which produces and excretes human parathyroid hormone, the method of the present invention may be applied to produce a plasmid containing DNA coding for parathyroid hormone from any species. Further, said plasmid may be inserted into any species of yeast. The invention thus is not limited to S. cerevisiae.

The cloned human parathyroid hormone produced by the yeast of the present invention has a variety of known and potential uses. For example, it is current medical theory that human parathyroid hormone will be

highly effective in treating osteoporosis. Genetically engineered parathyroid hormone may be useful in an analytical kit for measuring parathyroid hormone levels in humans and animals. Human parathyroid hormone or fragments thereof may also be used for treatment of humans or animals displaying reduced or pathologically altered blood calcium levels. It is anticipated that many other uses will be discovered when genetically engineered parathyroid hormone is available in large quantities, for example as a result of the present invention.

The invention has been described herein with reference to certain preferred embodiments.

Claims

1. A plasmid for insertion in yeast comprising a nucleotide sequence coding for production and secretion of parathyroid hormone, more particularly human parathyroid hormone wherein the nucleotide sequence comprises:

10 30 50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT

70 90 110
TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA

130 150 170
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT

190 210 230
TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

250 270 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG

310 330 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT

370 390 410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG

430 450 470
ATAAAAGAGAGGCTGAAGCTWSNGTNWSNGARATHCARYTNATGCAYAAYYTNGGNAARC

490 510 530
AYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTNCARGAYGTNCAYAAYT

550 570 590
TYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWSNCARMGNCCNMGNAARA

610 630 650
ARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGARGCNGAYAARGCNG

670 690 710
AYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAAATGAAAACAGATATTGTCAGAGT

730 750 770
TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAATATAC

790 810 830
TTTTCATTTCTCCGTAAACAACCTGTTTTCGCATGTAATATCCTTTTCTATTTTTCGTTT

850 870
CGTTACCAACTTTACACATACTTTATATAGCTAT,

wherein M = A or C; R = A or G; W = A or T; S = C or G; Y = C or T ; H = A or C or T; N = A or G or C or T, nucleotides 1 to 173 of said sequence making up the MF α 1 promoter region and 5' non-coding sequence; nucleotides 174 to 440 being the MF α 1 N-terminal coding sequence; nucleotides 441 to 695 being an HPTH sequence, nucleotides 696 to 726 being an HPTH 3' non-coding sequence from pSSHPTH-10 shown in Figure 6, nucleotides 727 to 732 being from pUC 19, nucleotides 733 to 874 being the MF α 1 3' non-coding sequence and transcriptional termination signal.

2. The plasmid of claim 1 wherein the nucleotide sequence comprises:

```
        10                  30                  50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT

        70                  90                  110
TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA

        130                 150                 170
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT

        190                 210                 230
TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

        250                 270                 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG

        310                 330                 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT

        370                 390                 410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG
```

```
        430                   450                   470
ATAAAAGAGAGGCTGAAGCTTCTGTGAGTGAAATACAGCTTATGCATAACCTGGGAAAAC

        490                   510                   530
ATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTGCAGGATGTGCACAATT

        550                   570                   590
TTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCCCAGAGGCCCCGAAAAA

        610                   630                   650
AGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGGAGAGGCAGACAAAGCTG

        670                   690                   710
ATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAAACAGATATTGTCAGAGT

        730                   750                   770
TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAATATAC

        790                   810                   830
TTTTCATTTCTCCGTAAACAACCTGTTTTCCCATGTAATATCCTTTTCTATTTTTCGTTT

        850                   870
CGTTACCAACTTTACACATACTTTATATAGCTAT,
```

wherein nucleotides 1 to 173 of said sequence make up the MF α 1 promoter region and 5' non-coding sequence; nucleotides 174 to 440 are the MF α 1 N-terminal coding sequence; nucleotides 441 to 695 are an HPTH sequence, nutcleotides 696 to 726 are an HPTH 3' non-coding sequence from pSSHPTH-10, shown in Figure 6, nucleotides 727 to 732 are from pUC 19, nucleotides 733 to 874 are the MF α 1 3' non-coding sequence and transcriptional termination signal.

3. A microorganism in the form of a yeast preferably Saccharomyces cerevisiae, containing the plasmid of claim 1 or 2.

4. A method of making the plasmid of claim 1 or 2, comprising the insertion of the nucleotide sequence of claim 1 or 2, coding for PTH, particularly human PTH, into a plasmid.

5. The method of claim 4, wherein the insertion is carried out by recloning pSSHPTH-10, the restriction map of which is shown in Fig. 6.

Patentansprüche

1. Plasmid zur Insertion in Hefe, umfassend eine Nucleotidsequenz, die für die Herstellung und Absonderung von Parathormon und insbesondere für menschliches Parathormon codiert, wobei die Sequenz folgende Nucleotide umfaßt:

```
          10                  30                  50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT

          70                  90                 110
TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA

         130                 150                 170
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT

         190                 210                 230
TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

         250                 270                 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG

         310                 330                 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT

         370                 390                 410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG

         430                 450                 470
ATAAAAGAGAGGCTGAAGCTWSNGTNWSNGARATHCARYTNATGCAYAAYYTNGGNAARC

         490                 510                 530
AYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTNCARGAYGTHCAYAAYT

         550                 570                 590
TYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWSNCARMGNCCNMGNAARA

         610                 630                 650
ARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGARGCNGAYAARGCNG

         670                 690                 710
AYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAAATGAAAACAGATATTGTCAGAGT
```

730 750 770

790 810 830

850 870

wobei M = A oder C; R = A oder G; W = A oder T; S = C oder G; Y = C oder T; H = A oder C oder T; N = A oder G oder C oder T, wobei die Nucleotide 1 bis 173 der Sequenz die MFα1-Promotorregion und die 5'-nicht-codierende Sequenz ausmachen, es sich bei den Nucleotiden 174 bis 440 um die MFα1-N-terminale codierende Sequenz handelt, es sich bei den Nucleotiden 441 bis 695 um eine HPTH-Sequenz handelt, es sich bei den Nucleotiden 696 bis 726 um eine HPTH-3'-nicht-codierende Sequenz aus pSSHPTH-10 handelt, das in Fig. 6 gezeigt ist, die Nucleotide 727 bis 732 aus pUC19 stammen und es sich bei den Nucleotiden 733 bis 874 um die MFα1-3'-nicht-codierende Sequenz und das transkriptionale Terminationssignal handelt.

2. Plasmid nach Anspruch 1, wobei die Sequenz folgende Nucleotide umfaßt:

10 30 50

AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT

70 90 110

TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA

130 150 170

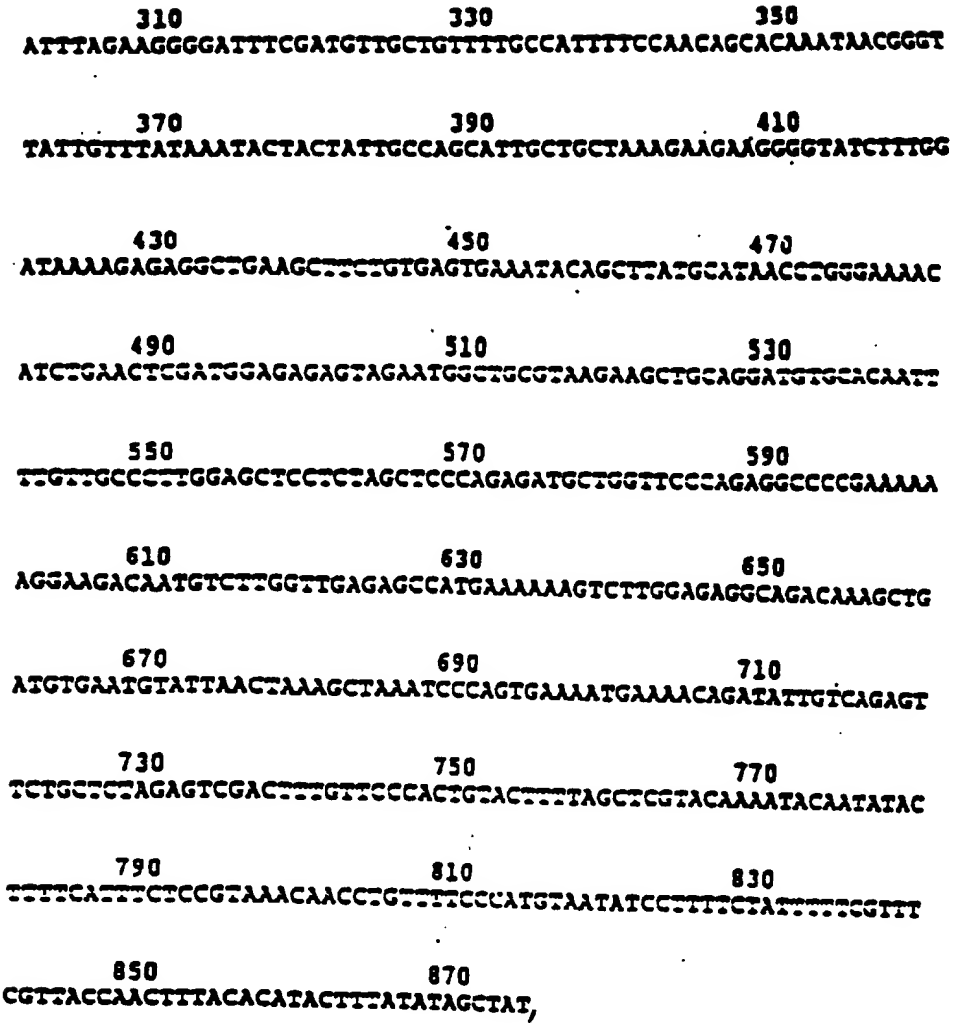
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT

190 210 230

TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

250 270 290

ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG



wobei die Nucleotide 1 bis 173 der Sequenz die MFα1-Promotorregion und die 5'-nicht-codierende Sequenz ausmachen, es sich bei den Nucleotiden 174 bis 440 um die MFα1-N-terminal codierende Sequenz handelt, es sich bei den Nucleotiden 441 bis 695 um eine HPTH-Sequenz handelt, es sich bei den Nucleotiden 696 bis 726 um eine HPTH-3'-nicht-codierende Sequenz aus pSSHPTH-10 handelt, das in Fig. 6 gezeigt ist, die Nucleotide 727 bis 732 aus pUC19 stammen, es sich bei den Nucleotiden 733 bis 874 um die MFα1-3'-nicht-codierende Sequenz und das transkriptionale Terminationssignal handelt.

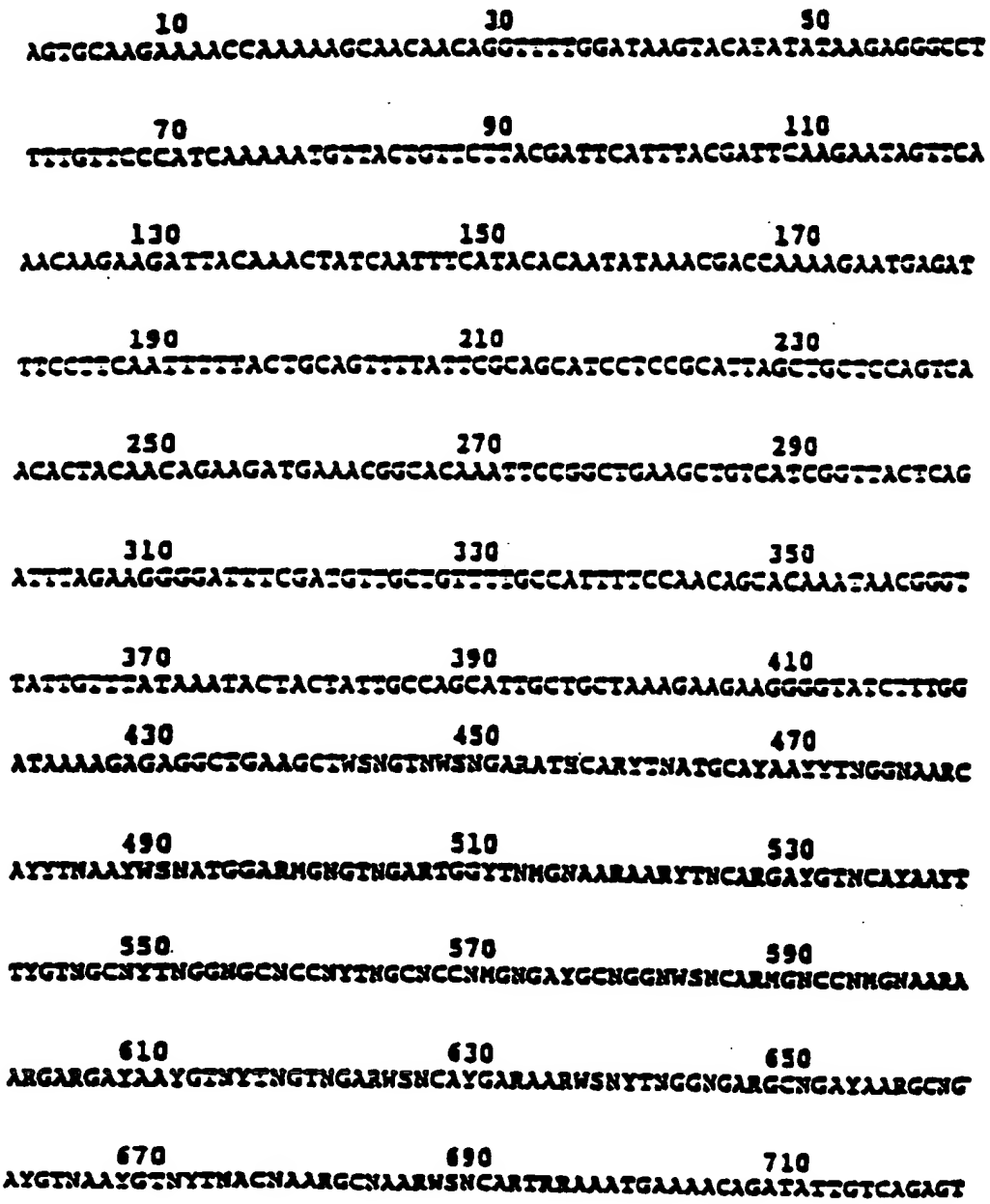
3. Mikroorganismus in Form einer Hefe, vorzugsweise Saccharomyces cerevisiae, enthaltend das Plasmid nach Anspruch 1 oder 2.

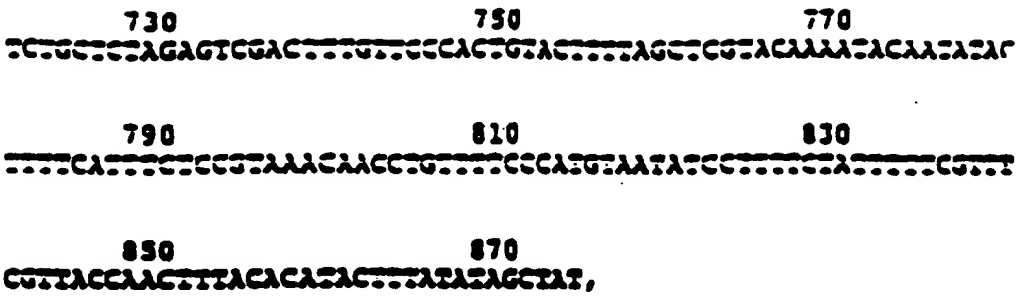
4. Verfahren zur Herstellung des Plasmids nach Anspruch 1 oder 2, umfassend die Insertion der Nucleotidsequenz von Anspruch 1 oder 2, die für PTH, insbesondere menschliches PTH codiert, in ein Plasmid.

5. Verfahren nach Anspruch 4, wobei die Insertion durch em ute Klonierung von pSSHPTH-10 durchgeführt wird, dessen Restriktionskart in Fig. 6 g zeigt ist.

Revendications

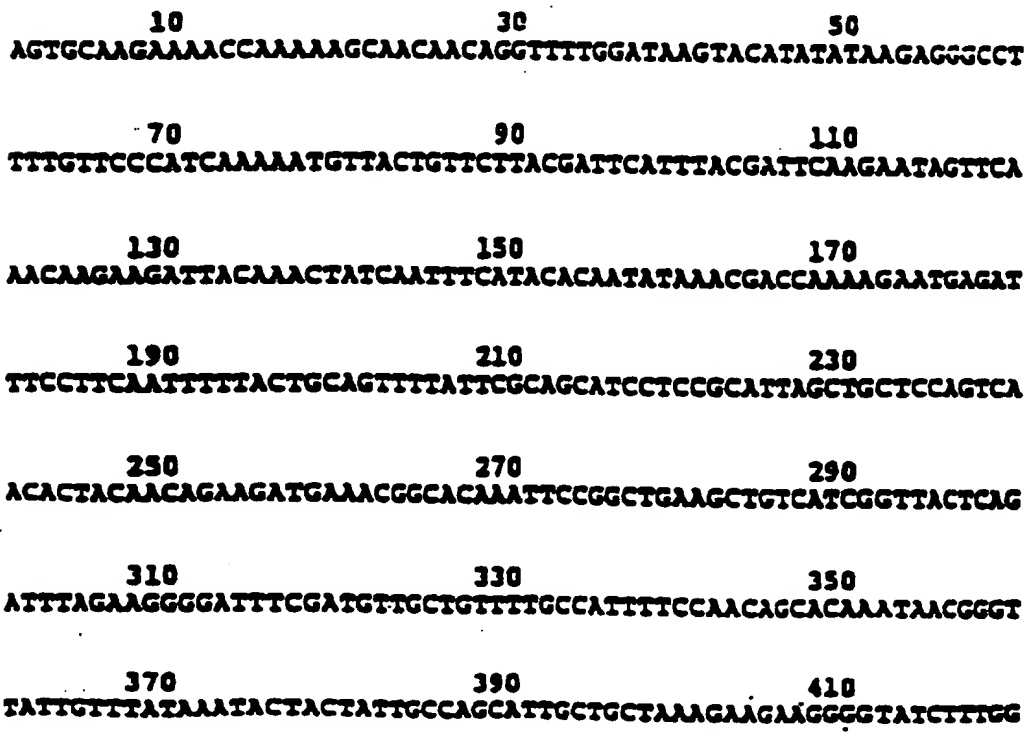
1. Plasmide pour une insertion dans une levure comportant une séquence nucléotidique codant pour une production et une sécrétion d'hormone parathyroïdienne, plus particulièrement d'une hormone parathyroïdienne humaine, dans lequel la séquence nucléotidique comporte :

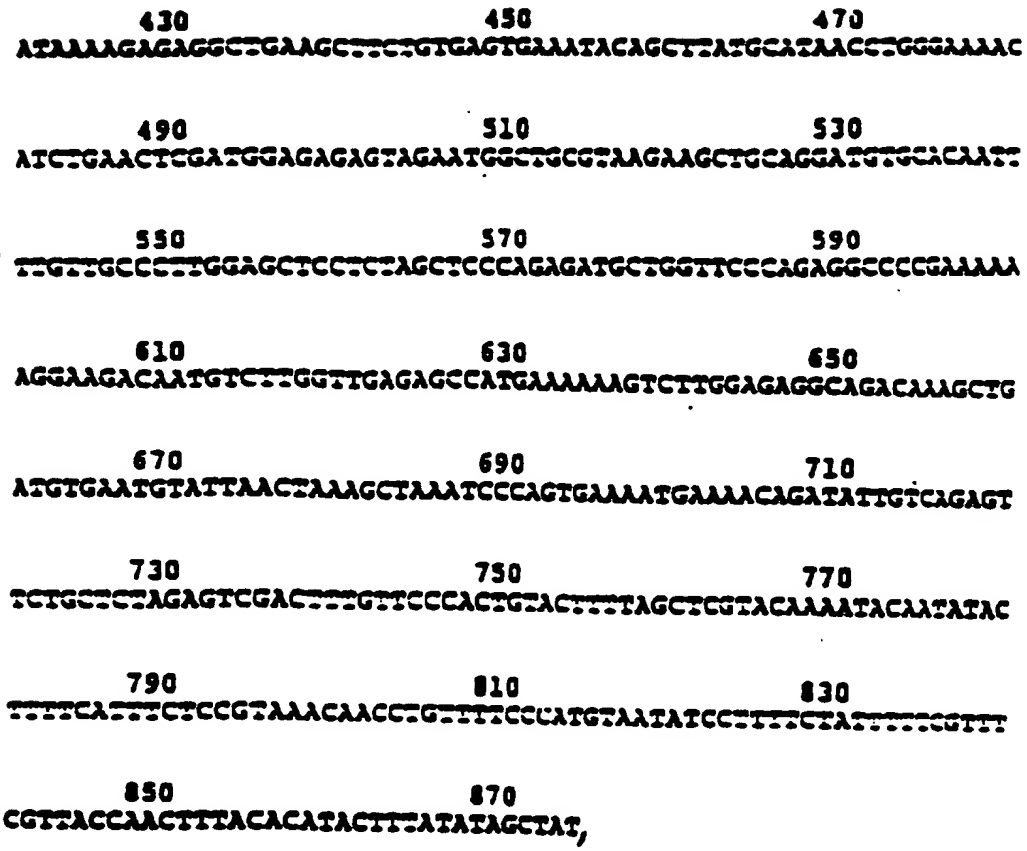




où M = A ou C; R = A ou G; W = A ou T; S = C ou G; Y = C ou T; H = A ou C ou T; N = A ou G ou C ou T, les nucléotides 1 à 173 de ladite séquence représentant la région du promoteur de MFα1 et la séquence 5' non codante; les nucléotides 174 à 440 étant la séquence codante N-terminale de MFα1; les nucléotides 441 à 695 étant une séquence de l'HPTH, les nucléotides 696à 726 étant une séquence 3' non codante de l'HPTH provenant de pSSHPTH-10 représenté sur la figure 6, les nucléotides 727 à 732 provenant de pUC19, les nucléotides 733 à 874 étant la séquence 3' non codante de MFα1 et le signal de terminaison de la transcription.

2. Plasmide selon la revendication 1, dans lequel la séquence de nucléotides comporte :





où les nucléotides 1 à 173 de ladite séquence représentent la région du promoteur de MFα1 et la séquence 5' non codante, les nucléotides 174 à 440 sont la séquence codante N-terminale de MFα1; les nucléotides 441 à 695 sont une séquence de l'HPTH, les nucléotides 696 à 726 sont une séquence 3' non codante de l'HPTH provenant de pSSHPTH-10 représenté sur la figure 6, les nucléotides 727 à 732 proviennent de pUC19, les nucléotides 733 à 874 sont la séquence 3' non codante de MFα1 et le signal de terminaison de la transcription.

3. Microorganisme d'une forme de levure, de manière préférentielle Saccharomyces cerevisiae, contenant le plasmide selon la revendication 1 ou 2.

4. Procédé pour réaliser le plasmide selon la revendication 1 ou 2, comportant l'insertion de la séquence nucléotidique selon la revendication 1 ou 2, codant pour la PTH, en particulier la PTH humaine, dans un plasmide.

5. Procédé selon la revendication 4, dans lequel l'insertion est réalisée en reclonant pSSHPTH-10, dont la carte de restriction est représentée sur la figure 6.

```
        10                  30                  50
ATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNATHTGYTTYYTN

        70                  90                  110
ACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNATGCAY

        130                 150                 170
AAYYTNGGNAARCAYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTNCAR

        190                 210                 230
GAYGTNCAYAAYTTYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWSNCAR

        250                 270                 290
MGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGAR

        310                 330
GCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTRR
```

M = A or C
R = A or G
W = A or T
S = C or G
Y = C or T
H = A or C or T
N = A or G or C or T.

FIGURE 1

```
        10                  30                  50
ATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTTT

        70                  90                  110
CTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTATG

        130                 150                 170
CATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTG

        190                 210                 230
CAGGATGTGCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCC

        250                 270                 290
CAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGGA

        310                 330
GAGGCAGACAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAGTGA
```

FIGURE 2

```
        10                  30                  50
TATGATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNATHTGYTT

        70                  90                  110
YYTNACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNAT

        130                 150                 170
GCAYAAYYTNGGNAARCAYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYT

        190                 210                 230
NCARGAYGTNCAYAAYTTYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWS

        250                 270                 290
NCARMGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGG

        310                 330                 350
NGARGCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAAATGAAA

        370                 390                 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC

        430
AAAGCTCTATTA
```

M = A or C
R = A or G
W = A or T
S = C or T
Y = C or T
H = A or C or T
N = A or G or C or T.

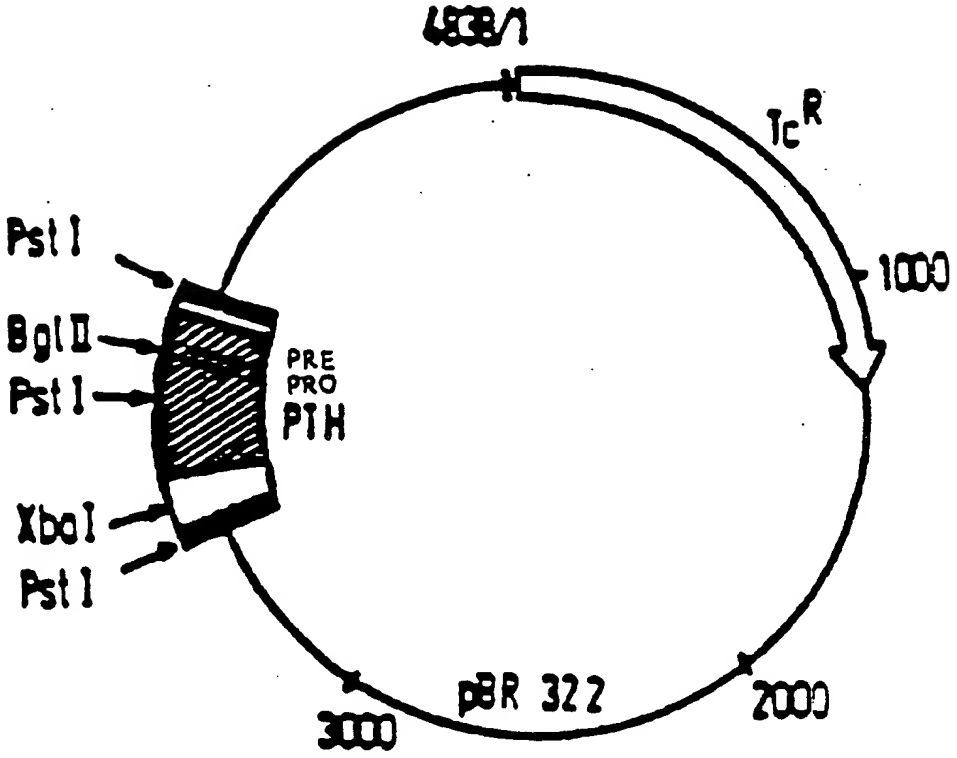
FIGURE 3

```
        10                  30                  50
TATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTT

        70                  90                  110
TCTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTAT

       130                 150                 170
GCATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCT

       190                 210                 230
GCAGGATGTGCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTC

       250                 270                 290
CCAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGG

       310                 330                 350
AGAGGCAGACAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAA

       370                 390                 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC

       430
AAAGCTCTATTA.
```

FIGURE 4

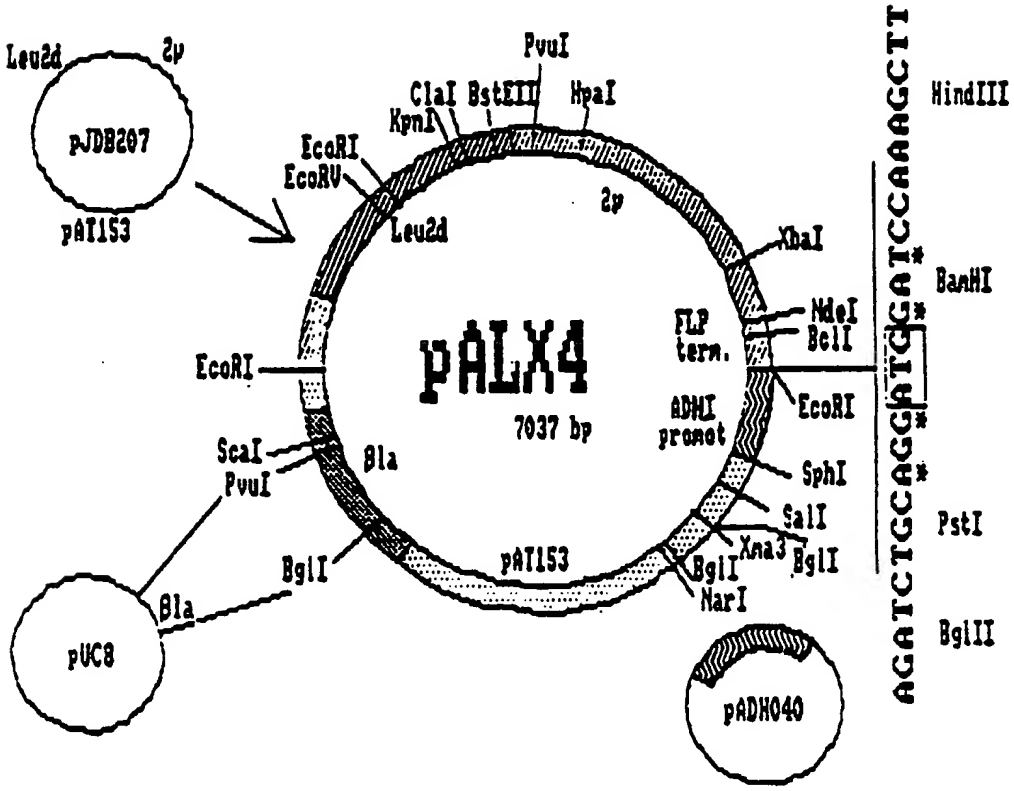
```
        10                  30                  50
TATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTT
    MetIleProAlaLysAspMetAlaLysValMetIleValMetLeuAlaIleCysPh

        70                  90                  110
TCTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTAT
eLeuThrLysSerAspGlyLysSerValLysLysArgSerValSerGluIleGlnLeuMe

       130                 150                 170
GCATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCT
tHisAsnLeuGlyLysHisLeuAsnSerMetGluArgValGluTrpLeuArgLysLysLe

       190                 210                 230
GCAGGATGTGCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTC
uGlnAspValHisAsnPheValAlaLeuGlyAlaProLeuAlaProArgAspAlaGlySe

       250                 270                 290
CCAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGG
rGlnArgProArgLysLysGluAspAsnValLeuValGluSerHisGluLysSerLeuGl

       310                 330                 350
AGAGGCAGACAAAGCTGATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAA
yGluAlaAspLysAlaAspValAsnValLeuThrLysAlaLysSerGlnEnd

       370                 390                 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC

       430
AAAGCTCTATTA.
```

FIGURE 5



pSSHPTH-10

FIGURE 6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PvuI
ClaI
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HpaI
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2μ
XbaI
NdeI
BclI
EcoRI
FLP term.
ADH1 promot
SphI
SalI
Xma3
BglI
NarI
pAT153
pALX4
7037 bp
EcoRI
ScaI
PvuI
Bla
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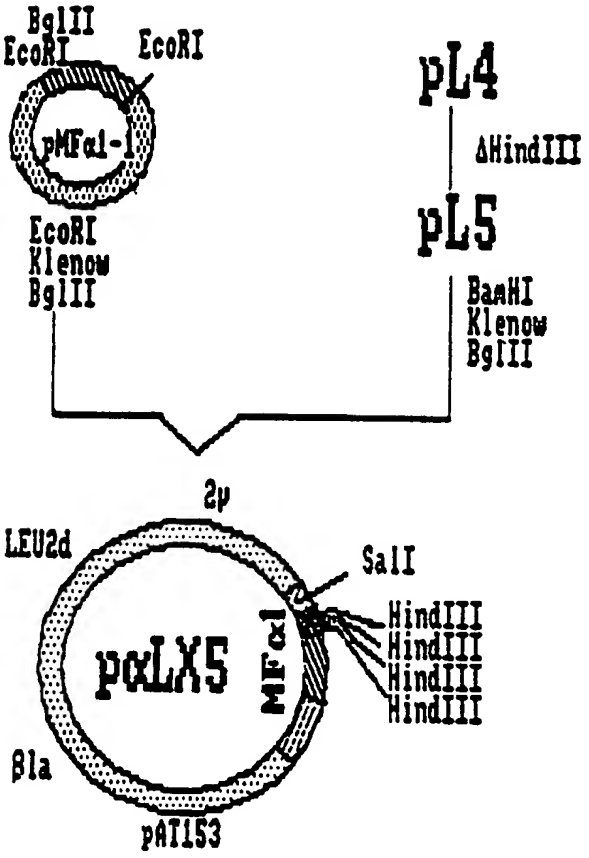
AGATCTGCAGGATGGATCCAAAGCTT
HindIII
BamHI
PstI
BglII

FIGURE 7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EcoRI
EcoRI
pMFα1-1
EcoRI
Klenow
BglII
pL4
ΔHindIII
pL5
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Klenow
BglII
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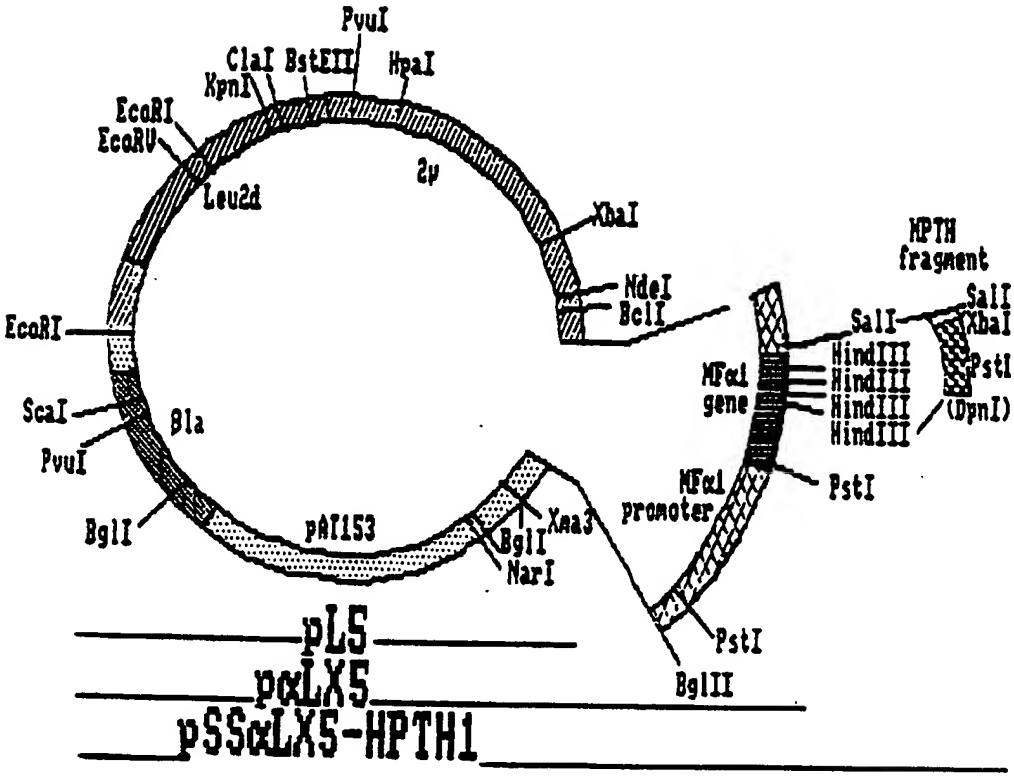
HindIII
pαLX5
MFα1
βla
pAT153

FIGURE 8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fragment
SalI
XbaI
PstI
(DpnI)
EcoRI
ScaI
Bla
PvuI
BglI
pAT153
Xaa3
BglI
NarI
MFα1
gene
HindIII
HindIII
HindIII
HindIII
SalI
MFα1
promoter
PstI
PstI
BglII
pLS
pαLX5
pSSαLX5-HPTH1

FIGURE 9

```
        10                  30                  50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT

        70                  90                  110
TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA

        130                 150                 170
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT

        190                 210                 230
TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

        250                 270                 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG

        310                 330                 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT

        370                 390                 410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG

        430                 450                 470
ATAAAAGAGAGGCTGAAGCTWSNGTNWSNGARATHCARYTNATGCAYAAYYTNGGNAARC

        490                 510                 530
AYYTNAAYWSNATGGARMGNGTNGARTGGYTNMGNAARAARYTNCARGAYGTNCAYAAYT

        550                 570                 590
TYGTNGCNYTNGGNGCNCCNYTNGCNCCNMGNGAYGCNGGNWSNCARMGNCCNMGNAARA
```

FIGURE 10

610 630 650
ARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGARGCNGAYAARGCNG

670 690 710
AYGTNAAYGTNYTNACNAARGCNAARWSNCARTRRAAATGAAAACAGATATTGTCAGAGT

730 750 770
TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAATATAC

790 810 830
TTTTCATTTCTCCGTAAACAACCTGTTTTCCCATGTAATATCCTTTTCTATTTTTCGTTT

850 870
CGTTACCAACTTTACACATACTTTATATAGCTAT, wherein

M = A or C
R = A or G
W = A or T
S = C or G
Y = C or T
H = A or C or T
N = A or G or C or T

FIGURE 10 (Cont.)

```
        10                  30                  50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT

        70                  90                 110
TTTGTTCCCATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA

       130                 150                 170
AACAAGAAGATTACAAACTATCAATTTCATACACAATATAAACGACCAAAAGAATGAGAT

       190                 210                 230
TTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

       250                 270                 290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAG

       310                 330                 350
ATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT

       370                 390                 410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG

       430                 450                 470
ATAAAAGAGAGGCTGAAGCTTCTGTGAGTGAAATACAGCTTATGCATAACCTGGGAAAAC

       490                 510                 530
ATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTGCAGGATGTGCACAATT

       550                 570                 590
TTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCCCAGAGGCCCCGAAAAA
```

FIGURE 11

```
      610                   630                   650
AGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGGAGAGGCAGACAAAGCTG

      670                   690                   710
ATGTGAATGTATTAACTAAAGCTAAATCCCAGTGAAAATGAAAACAGATATTGTCAGAGT

      730                   750                   770
TCTGCTCTAGAGTCGACTTTGTTCCCACTGTACTTTTAGCTCGTACAAAATACAATATAC

      790                   810                   830
TTTTCATTTCTCCGTAAACAACCTGTTTTCCCATGTAATATCCTTTTCTATTTTTCGTTT

      850                   870
CGTTACCAACTTTACACATACTTTATATAGCTAT
```

FIGURE 11 (Cont.)

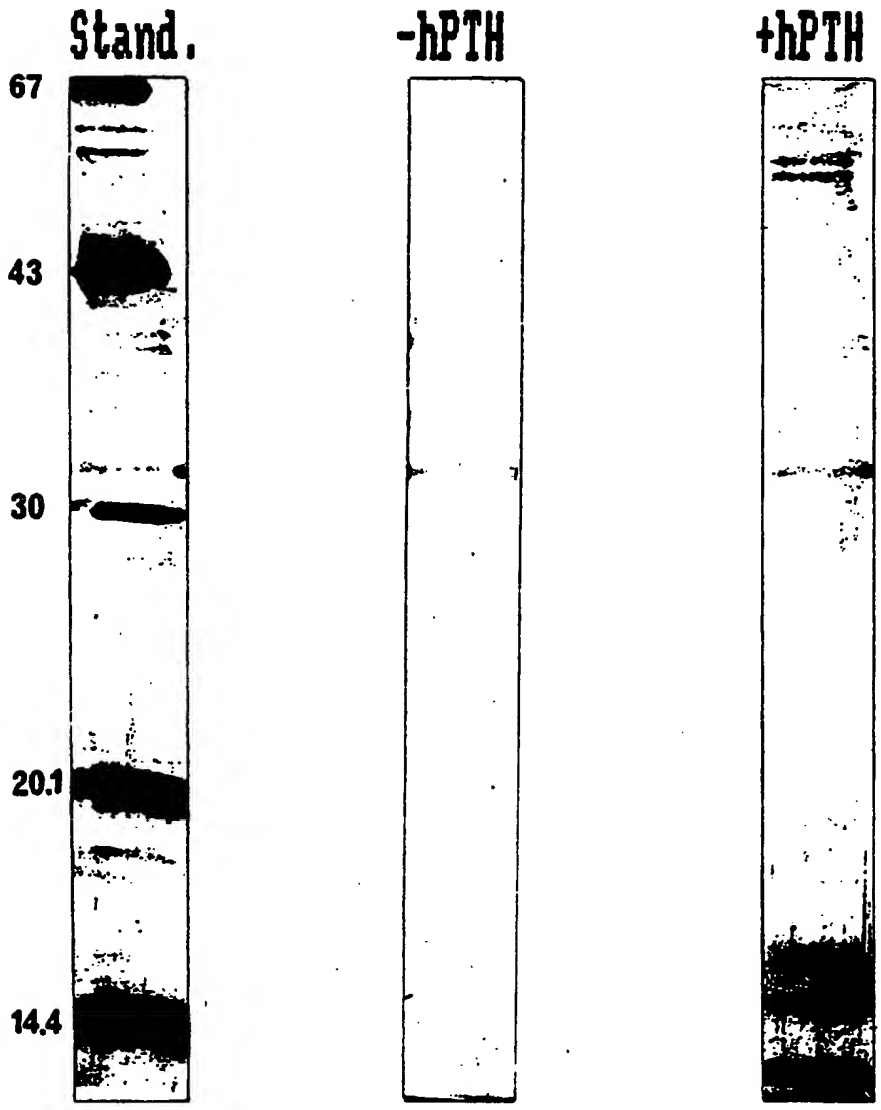

Fig. 12